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I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

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Patents Form 1/77

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1/77

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P01/7700 0.00-0113121.8

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

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Cardiff Road
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1. Your reference — MRH/P16237

2. Patent application number *(The Patent Office will fill in this part)* — **0113121.8** — 30 MAY 2001

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

University of Leeds
LEEDS
LS2 9JT

6243554002

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — BIOLOGICALLY ACTIVE PHOTOSENSITISERS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

~~Harrison Goddard Foote~~
~~Tower House~~
~~Merrion Way~~
~~LEEDS~~
~~LS2 8PA~~
~~UK~~

PHOTOPHARMICA LIMITED
LEEDS INNOVATION CENTRE
103 CLARENDON ROAD
LEEDS
LS2 9JT

Patents ADP number *(if you know it)* — ~~14571001~~

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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
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c) any named applicant is a corporate body.
See note (d))

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Continuation sheets of this form	
Description	12
Claim *(s)*	
Abstract	
Drawing *(s)*	6 + 6

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature	Date
Harrison Goddard Foote	29-05-01

12. Name and daytime telephone number of person to contact in the United Kingdom — Michael Harrison — 0113 2901400

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BIOLOGICALLY ACTIVE PHOTOSENSITISERS

Field of the Invention

This invention relates to biologically active photosensitisers which are strongly photocytotoxic and have application in the areas of photodynamic therapy and photosterilisation.

Background to the Invention

It is known that certain organic compounds ("*photosensitisers*") can induce cell death by absorption of light in the presence of oxygen. The cytotoxic effect involves *Type I* and/or *Type II* photooxidation. Such photosensitisers find use in the treatment of cancer and other diseases with light (*photodynamic therapy)* and in the sterilisation of surfaces and fluids by the light-induced destruction of microbes.

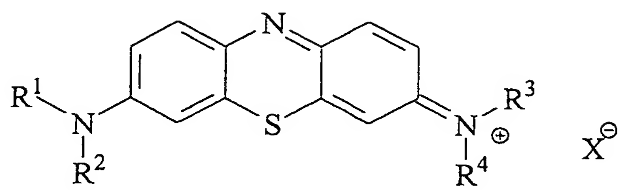
It is also known that certain coloured phenothiazinium compounds, (*e.g.* Methylene Blue) can take part in Type I and Type II photooxidation processes, but, with a few exceptions, compounds of this type thus far have proved ineffective or of low efficacy as sensitisers for photodynamic therapy, or have shown low photochemical antimicrobial activity.

For application in photodynamic therapy, a good sensitiser must have certain specific properties. Most importantly, it should show no dark toxicity, it should accumulate in tumour cells and cause their destruction efficiently on exposure to light (preferably wavelengths *ca.* 600 –800 nm), and it should not cause skin photosensitivity in the patient.

For applications in photo-sterilisation, a good sensitiser must show a strong phototoxic effect to a wide range of microrganisms, ideally using ambient light, and should not photobleach readily.

Statements of the Invention

According to the present invention, there is provided a phenothiazinium compound by Formula (I):



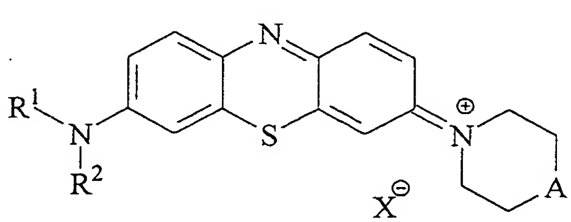
(I)

wherein:

R^1, R^2,R^3, R^4 are saturated alkyl chains (same or different) of the general formula C_nH_{2n+1}, where n can have the value 1-6, and the chain may be linear or branched, and where X^- represents a monoanion, which may be Cl^-, Br^-, I^-, F^-, NO_3^-, HSO_4^-, $CH_3CO_2^-$ or a dianion, namely, SO_4^{2-} or $H_2PO_4^{2-}$,

but excluding structures where R^1, R^2,R^3, R^4 (same or different) are each CH_3 and/or CH_3CH_2,

or the Formula (II)



(II)

wherein:

R^1, R^2 are saturated alkyl chains (same or different) of the general formula C_nH_{2n+1}, where n can have the value 1-6, and the chain may be linear or branched, A is CH_2, NH, NAlkyl or O, and where X^- represents a monoanion, which may be Cl^-, Br^-, I^-, F^-, NO_3^-, HSO_4^-, $CH_3CO_2^-$ or a dianion, namely SO_4^{2-} or $H_2PO_4^{2-}$.

The present invention also provides salts and derivatives of compounds of the Formulae I and II.

Furthermore, the present invention provides a conjugate or composite formed between a compound of Formula I or Formula II and a polymer. Preferably the bond between the compound of Formula I or II and the polymer is a covalent bond. Preferred polymers include those having anhydride and ester groups.

In addition, the present invention provides a compound formed by the reaction between a compound of Formula I or Formula II and a chlorotriazine derivative. The chlorotriazine derivative may be a polymer having chlorotriazine groups attached thereto.

The compounds of the present invention are useful as photosensitising drugs for photodynamic therapeutic treatment of conditions such as cancer, precancerous disease, macular degeneration, vascular problems such as artherosclerosis and restenosis, and rheumatoid arthritis. Specific advantages of these materials relate to their low tendency to colour skin and their low tendency to sensitise skin to ambient light when administered systemically. The compounds may also be used as anti-microbial treatments for skin and other local infections, for sterilisation of burn wounds and other lesions, and for the treatment of dental bacterial disease.

The said compounds are also useful as photoactivated antimicrobial agents for general sterilisation of surfaces and fluids. Specific advantages of these compounds

over existing known antimicrobial photosensitisers are their high photocytotoxicity, combined with a low tendency to undergo photobleaching.

Accordingly, the present invention also provides compositions comprising a compound of the present invention together with a diluent or excipient.

As indicated above, compounds of the present invention can be used in various methods. For instance, the present invention also provides a method of treatment of a condition such as cancer, pre-cancerous disease, macular degeneration and vascular problems such as arterosclerosis and restinosis, and rheumatoid arthritis, the method including employing a compound of the invention in a photodynamic therapeutic treatment.

Accordingly, the present invention provides a method of treatment of microbial infections, burn wounds and other lesions and of dental bacterial disease, the method comprising applying to the area to be treated a therapeutically effective amount of a compound of the present invention and exposing said area to light to render active said compound.

Furthermore, the present invention also provides a method of sterilising a surface or a fluid comprising applying the compound of the present invention to said surface or fluid and activating said compound by means of light.

Appropriate compounds of the present invention may be attached to polymeric surfaces, permanently by covalent bonds or reversibly by intermolecular interactions, thus affording a surface that can be sterilised whenever required by the application of light. This would be useful, for example, with intravenous lines in patients, where maintaining long-term sterility of the relevant surfaces is problematical. The resistance of the compounds to photobleaching is an advantage in such applications, where prolonged stability of the chromophore is required.

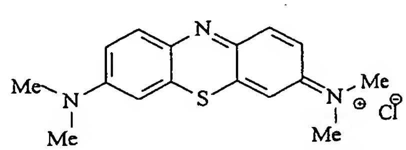
Detailed description of the invention

(A) Photodynamic activity

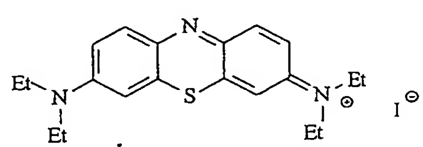
Figure 1 of the accompanying drawings shows the anti-tumour photodynamic efficacy (% tumour necrosis) of symmetrically substituted thiazines of type (I). Female CBA/Gy mice with CaNT subcutaneous tumours were injected with a solution of the drug at a dose of 16.6 μmol per kilogram. They were treated with the appropriate wavelength of light 1 hour after injection. The light source was a Patterson lamp with appropriate filters giving a bandwidth of 30 nm, and treatment was 60 J cm^{-2} at a rate of 50 mW cm^{-2}. Wavelengths examined were 610, 660 and 685 nm. Each bar in Figure 1 represents the mean of three mice ± s.e.

Methylene Blue, Ethylene Blue, and the tetra-n-propyl derivative were applied as 1.67 mmolar solutions in saline solution, whereas all the other drugs, and also the tetra-n-propyl derivative, were applied as 1.67 mmolar solutions in a mixture of dimethylsulphoxide (2%) and water (98%).

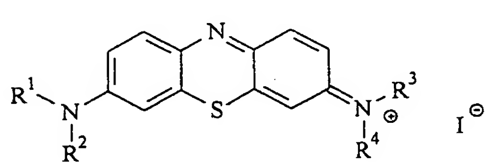
Compounds examined:



Methylene Blue



Ethylene Blue



$R^1 - R^4$ = n- C_3H_7 : tetra-n-propyl

$R^1 - R^4$ = n- C_4H_9 : tetra-n-butyl

$R^1 - R^4$ = n- C_5H_{11} : tetra-n-pentyl
$R^1 - R^4$ = n- C_6H_{13} : tetra-n-hexyl

It can be seen that tumour response is very dependent on the nature of the alkyl groups, and the tetra-n-pentyl and tetra-n-butyl derivatives were particularly effective versus Methylene Blue and Ethylene Blue.

Table 1 shows the wavelength band at which the best tumour response was observed and λ_{max} measured in water & cells.

Table 1

Thiazine	**λ_{max} (1μM in H_20)**	**λ_{max} cells ± SD**	**Optimum λ *in-vivo***
Butyl	677	634 ± 8	660 ± 15
Pentyl	679	643 ± 5	685 ± 15
Hexyl	685	615 ± 7	660 ± 15

The butyl, and pentyl derivatives were assessed in triplicate at different drug to light intervals at the following parameters and compared to the sensitiser PHP (the sensitiser most widely used clinically) at 8.35 μmol kg^{-1},630 ± 15:-
tetra-n-butyl: 16.7 μmol kg^{-1}, 660 ± 15
tetra-n-pentyl: 8.35 μmol kg^{-1},685 ± 15

After determining the best parameters for each sensitiser, the relative tumour responses under optimal conditions were as shown in Figure 2 of the accompanying drawings.

Thus the tetra-n-pentyl derivative is closely similar to PHP in its PDT activity.

Skin sensitisation was measured as the change in ear thickness following exposure to a solar simulator at 24 h post drug injection. Light exposure was repeated at 14 days

post drug injection to assess persistence of an induced skin photosensitivity. The thiazine sensitisers were compared with PHP. The results are shown in Figure 3. Each bar represents the mean of at least three mice ± s.e. The therapeutic dose used was 16.7 μmol kg^{-1} for butyl and 8.35 μmol kg^{-1} for pentyl and PHP.

The experiments were repeated two weeks after administering the sensitisers , with the results as shown in Figure 4.

It is evident that the tetra-n-pentyl phenothiazinium derivative has similar PDT activity to the commercial sensitiser PHP, but has a much lower tendency to cause skin photosensitisation, even when only 24 hours have elapsed after administration. Thus such a sensitiser has the potential to overcome the problem of skin photosensitisation in patients, which is a major problem associated with PHP, MTHPC and other sensitisers in current use.

(B) <u>Photo-antimicrobial Activity</u>

<u>E.coli Cell Kill</u>

100ml of nutrient broth was innoculated with E.coli strain DH5, and incubated in a shaking incubator overnight at 37°C. Following incubation 10ml of the culture was transferred to 200ml of nutrient broth, and grown until in log phase. Cells were collected by centrifugation (3000rpm, 10 minutes), and resuspended in 0.1M potassium phosphate buffer (pH7.0) and centrifuged again (3000rpm, 10 minutes). The supernatant was discarded and the pellet resuspended in 0.1M potassium phosphate buffer (pH7.0) to an absorbance of 0.85-0.90 at 650nm. 25ml of the cell suspension was incubated with 0.25mls of a 0.1mM photosensitiser, for 30 minutes in the dark in a 37°C shaking incubator. The suspension was irradiated by a 500W halogen security lamp, from a distance of 75cm, for 60 minutes, the power of the lamp was 1.3mW/cm2 giving 4.68J/cm2 over the hour illumination. 50ml samples of the suspension were removed and diluted. 50ml of the diluted suspension was then

plated on nutrient agar and incubated overnight at 37°C to give colony forming units between 30-300. Cell kill was then measured.

Figure 5 shows the percentage of cells surviving for each of the symmetrical phenothiazine sensitisers (I), as a function of light exposure time.

It is clear that there is a pronounced dependence of photocytotoxicity on the nature of the alkyl chains in the phenothiazine, with the symmetrical tetra-n-butyl derivative showing the highest activity.

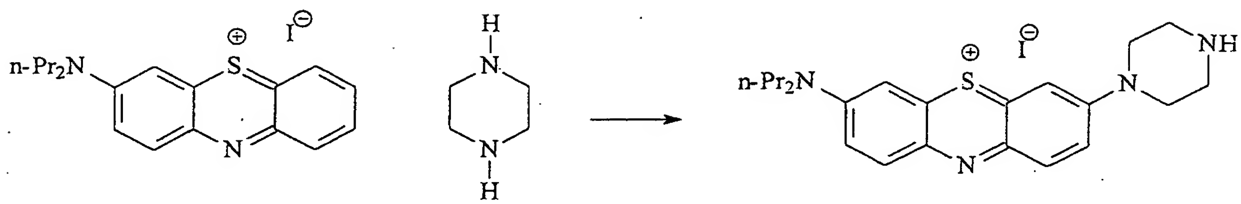
(C) Resistance to Photobleaching

To measure the resistance of the tetra-n-butyl derivative to photobleaching, photofading was carried out in (a) 60% methanol 40% 0.1M pH7 potassium phosphate buffer with 10uM of the dye (initially made up to 1mM in DMSO), and (b) an identical formulation but with addition of 100uM tryptophan. A similar solution with Methylene Blue was made up for comparison purposes. The solutions were illuminated at 9mW/cm2 with white light, when the Methylene Blue showed a high degree of photobleachong after 60 minutes, in contrast to the tetra-n-butyl derivative, which showed no detectable photobleaching after this time. The results are summarised in Figure 6 for the solutions without tryptophan. Identical results were obtained for the tryptophan-containing solutions.

(D) Phenothioazines of structure II suitable for attachment to, or adsorption on, polymer surfaces

Example IIa

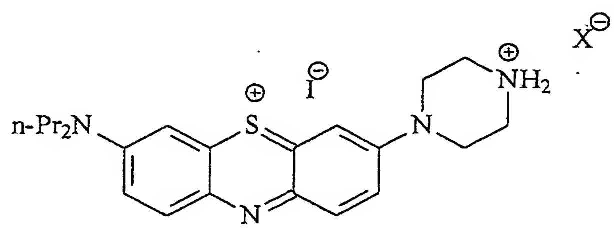
Phenothiazine IIa was made according to the following reaction scheme and was isolated as a dark blue solid. It was characterised by mass spectrometry.



IIa

Salt IIb

Compound (IIa) was extremely basic and readily protonated in dilute acids to give (IIb), which could be adsorbed strongly on polymeric surfaces, e.g. polyamides, polyacrylates, polyesters, polycarbonates, polyurethanes, and strongly resisted removal by water or solvents. Alternatively **IIa** could be adsorbed directly onto acidic surfaces to give **IIb** directly.



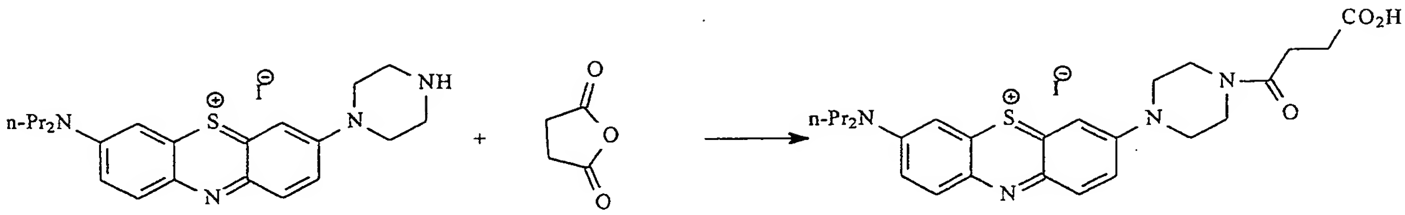
IIb

Attachment of the phenothiazine sensitisers to polymer substrates

Derivative IIa

Compound **IIa** proved very reactive as a nucleophile in substitution reactions that provide a means of attaching the sensitiser unit covalently to polymers.

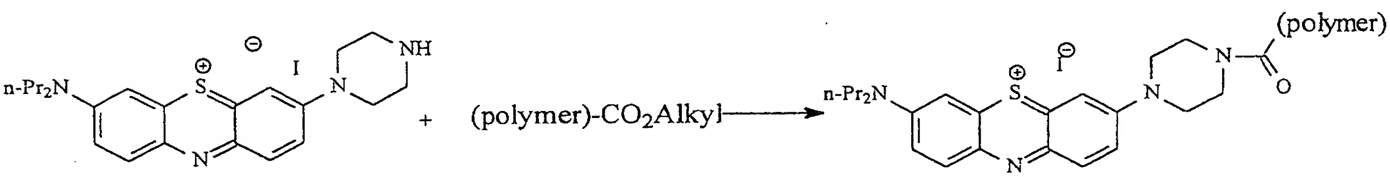
Thus reaction with anhydrides occurred, as exemplified by the following reaction:



IIc

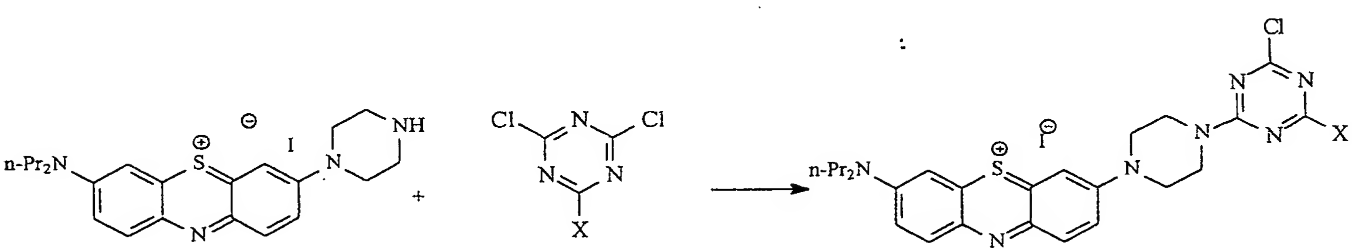
Compound **IIc** has been isolated and characterised by mass spectrometry, and exhibits typical photosensitising properties. Thus attachment of the sensitiser moiety to anhydride containing polymers can be carried out by an analogous reaction.

A similar nucleophilic substitution reaction will occur with polymers containing ester



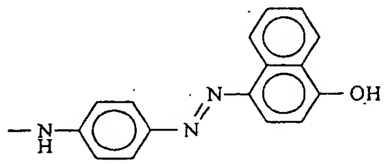
groups, i.e. :

Phenothiazine **IIa** is also very reactive towards chlorotriazine derivatives, as exemplified by the following reaction:



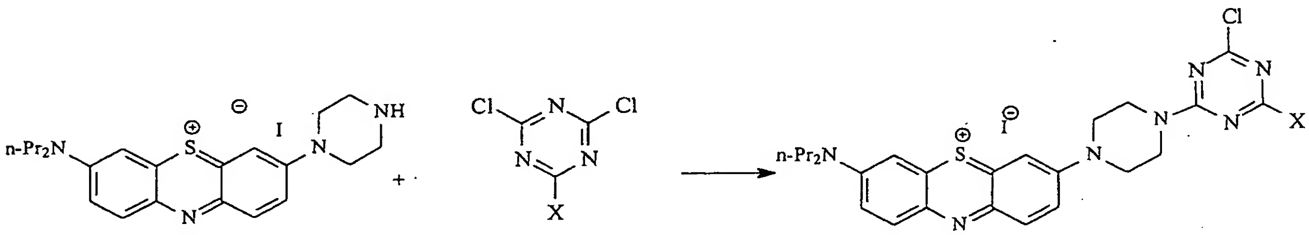
IId

Thus compound **IId** has been prepared, where X is:



This compound was characterised by mass spectrometry and shows the usual photosensitising characteristics of this class of sensitiser.

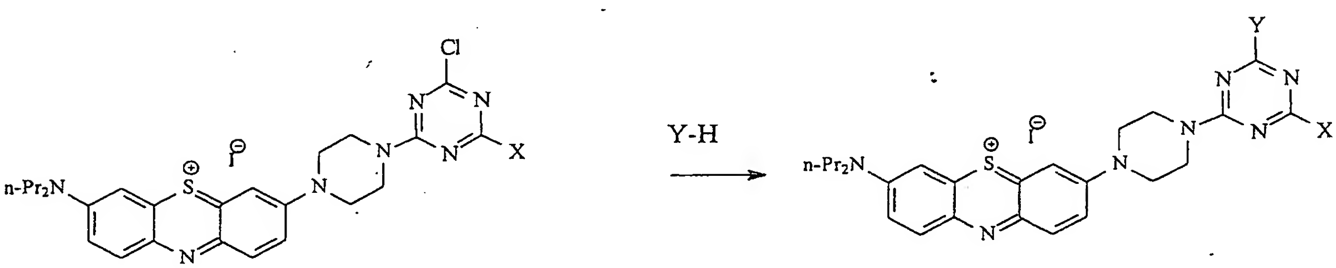
Thus linkage of **(IIa)** to polymers by a similar procedure can occur as follows:



Where X = -NH-(Polymer) in the case of polyamide polymers

X = -O-(Polymer) in the case of cellulosic polymers

Alternatively, the reactivity of the residual chlorine in the previous example can be exploited, as in the following reaction:



Where X and Y = -NH-(polymer)

Or X and Y = -O-(polymer)

Or X may be an amine –NHR or –NRR', and Y = -NH-(polymer), or –O-(polymer)

These are not the only means of attaching the phenothiazines to polymers, and other methods may by employed based on existing polymer-grafting chemistry known to those skilled in the art.

Such covalently linked phenothiazine-polymer composites are proposed as materials capable of long-term photosterilisation with ambient light, with resistance to photobleaching.

P16237.1

Figure 1. Comparison of the PDT activities of phenothiazinium sensitisers

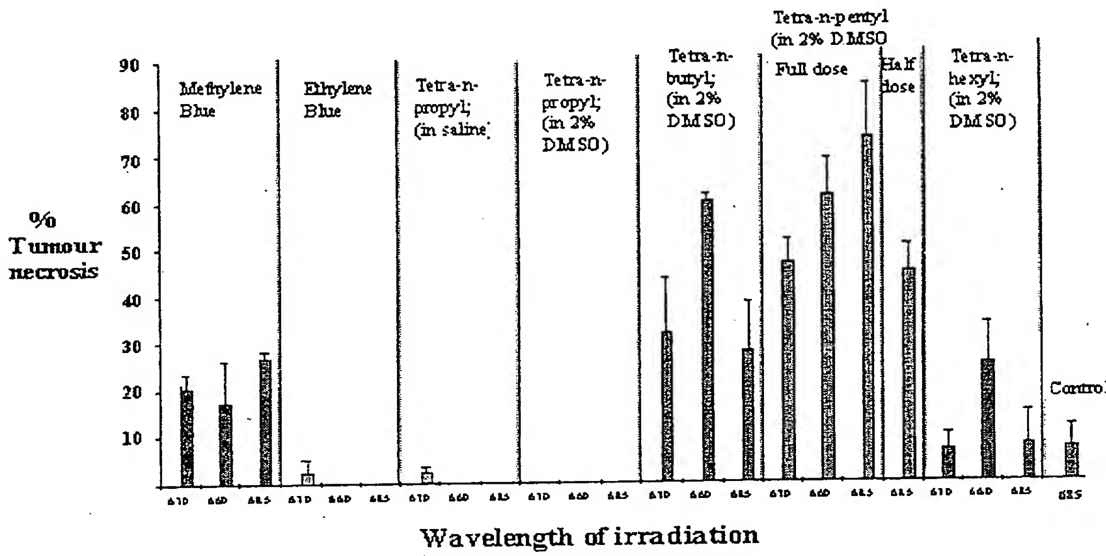


Figure 2 Tumour response at best parameters for each drug

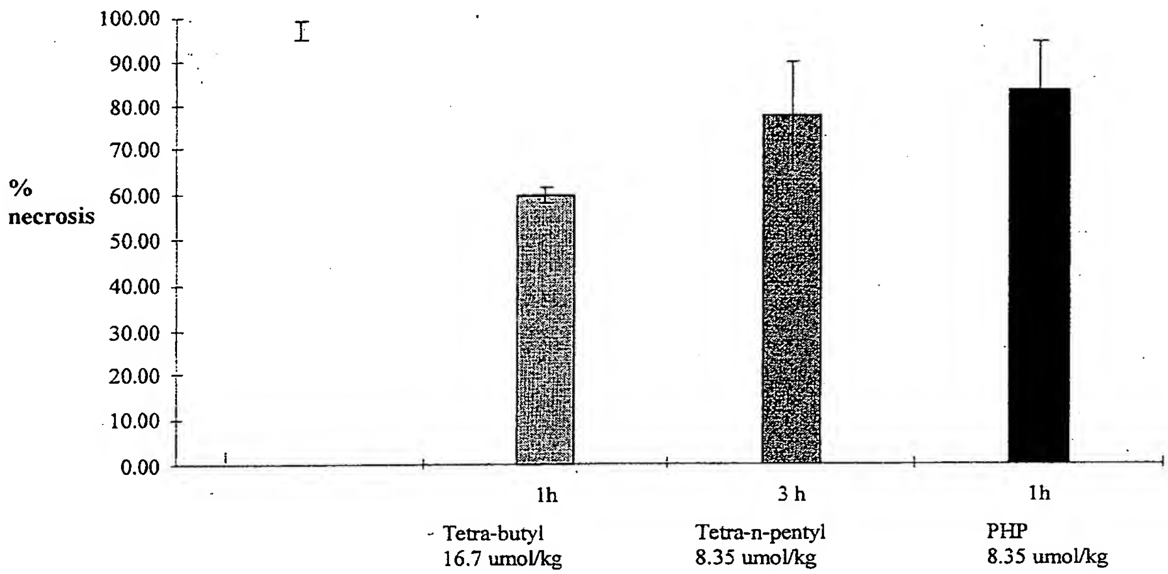


Figure 3 Change in ear thickness at 24h following initial light exposure (drug dose 16.7 μmol kg⁻¹).

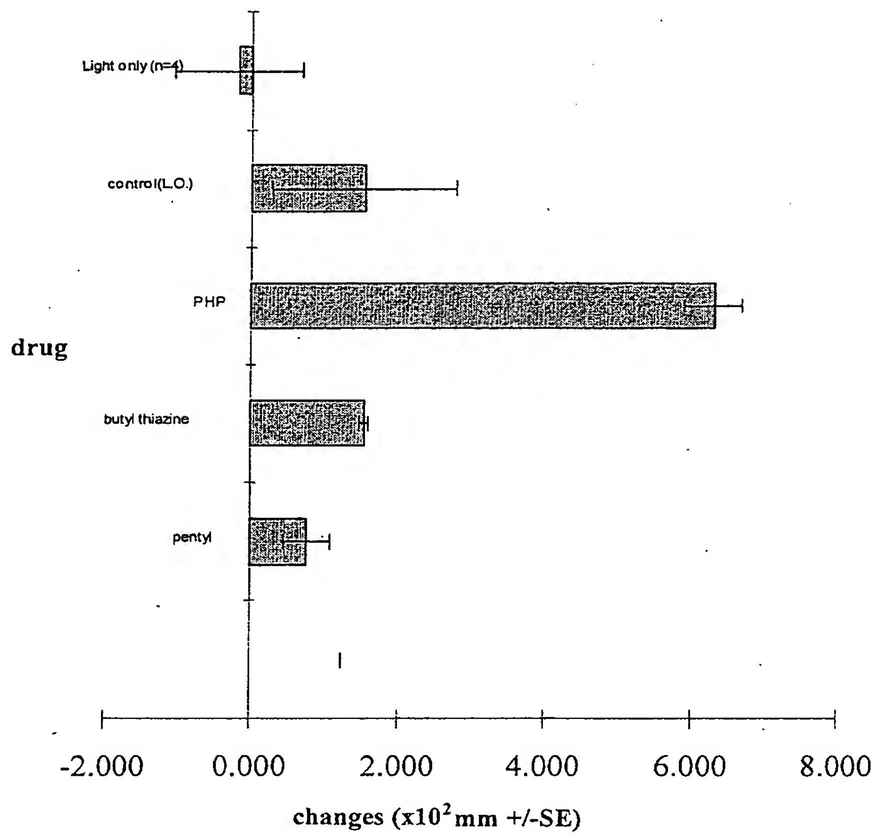


Figure 4. Relative skin photosensitisation activities of various sensitisers after 24 hours and after 2 weeks.

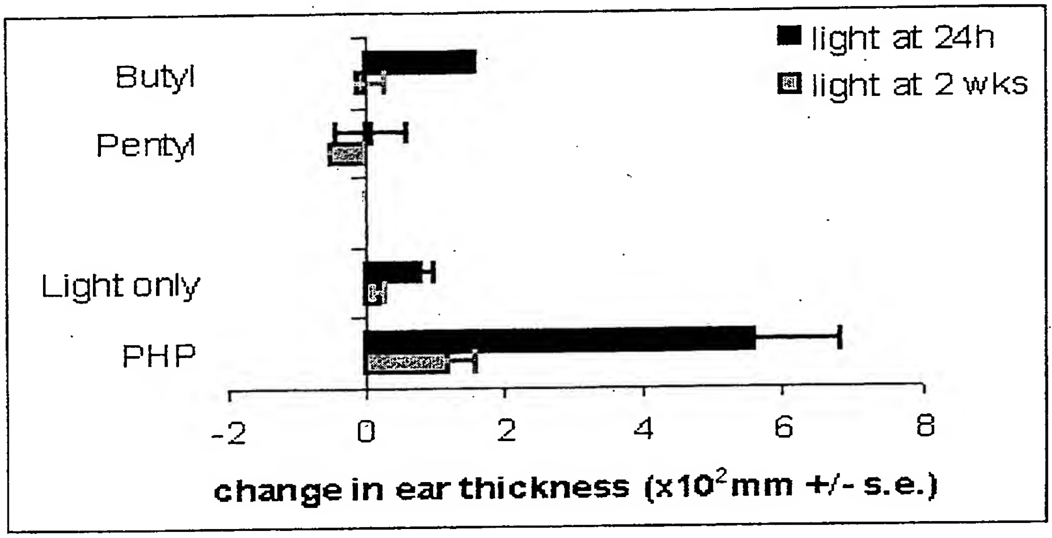


Figure 5: Percentage cell survival of E.coli following incubation with 10 μM phenothiazine and illumination at 1.3mW/cm²

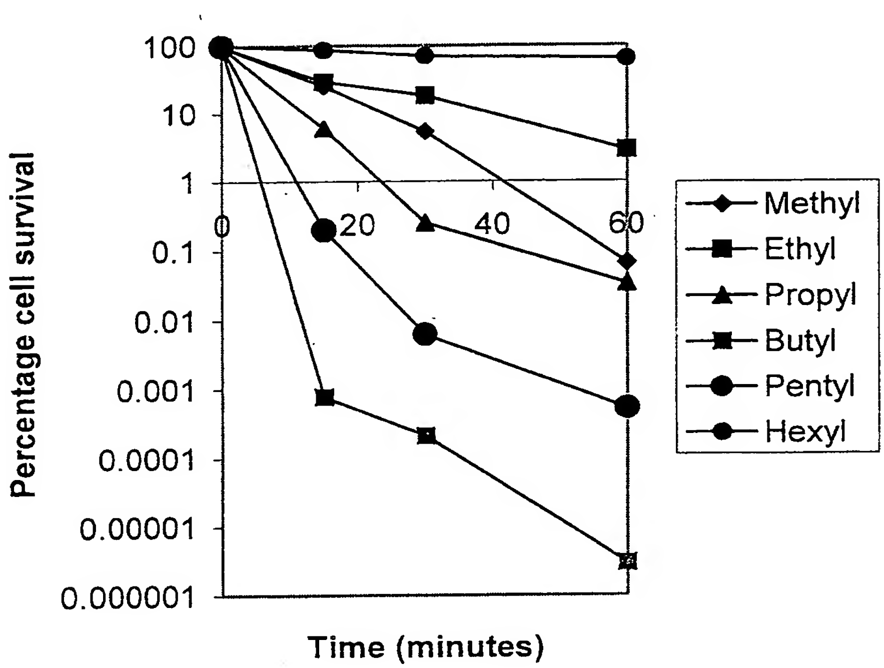


Figure 6: Absorbance maxima of Methylene blue and Tetra butyl phenothiazine following illumination at 9mW/cm²